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ONCOMELANIA HUPENSIS (GASTROPODA: RISSOOIDEA), INTERMEDIATE HOST OF *SCHISTOSOMA JAPONICUM* IN CHINA: GENETICS AND MOLECULAR PHYLOGENY BASED ON AMPLIFIED FRAGMENT LENGTH POLYMORPHISMS

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ABSTRACT

Schistosomiasis japonica is one of the most serious parasitic diseases in China. Subspecies of the pomatiopsid snail species *Oncomelania hupensis* transmit the human blood fluke *Schistosoma japonicum,* the parasite causing the disease. In at least one study involving the subspecies *Oncomelania hupensis robertsoni*, there was no clear phylogenetic concordance between the mitochondrial and nuclear data, and it was strongly recommended that future studies incorporate more data from nuclear loci in order to better understand phylogeography and host-parasite coevolution (Wilke et al., 2006). In this paper, we explore genetic diversity based AFLP data involving 25 populations throughout mainland China. AFLP is a DNA fingerprinting technique that detects the polymorphism of the whole genome without prior knowledge of the nucleotide sequence, with the high degree of reproducibility and small amount of template DNA needed.

Our AFLP data show that: (1) the genetic diversity within an *O. hupensis* population increases gradually with snail sample size, and when the sample size is more than 30 individuals, the genetic variation within one *O. hupensis* population trends to stabilization. (2) There is high intra-population genetic variation on the mainland of China, and these intra-population genetic variations from different areas differ significantly. (3) The considerable genetic differentiation occurs throughout China. (4) The genetic variation among populations of *O. h. hupensis* is higher than that of *O. h. robertsoni.* (5) The patterns of genetic differentiation are basically consistent with geographical distribution of snail populations. Our results are similar to the results of allozymes and COI gene sequences, excluding a snail population from Guangxi Province (Gx-1), indicating that there are three distinct subspecies in mainland China, namely *O. h. hupensis*, *O. h. robertsoni*, and *O. h. tangi*. However, our results do not support that the snail population from Guangxi Province belongs to *O. h . hupensis*.

Key words: *Schistosomiasis japonicum*, *Ocomelania hupensis*, China, molecular genetics, phylogeny.

INTRODUCTION

Schistosomiasis japonica is one of the most serious parasitic diseases in China, with a documented history of over 2100 years. By the end of 2003, an estimated 843,011 people were infected with *S. japonicum*. Among them, there were 1,114 cases with acute schistosomiasis and 24,441 cases suffering from advanced schistosomiasis. The human population at risk of infection was estimated around 65 million, and there were 74,000 infected cattle. The intermediate snail host, *Oncomelania hupensis,* infested an area of 3,787 km^2 (Zhou et al., 2005a). Basic epidemiological and malacological research were discouraged in China as the theoretical goal of preventing *schistosomiasis japonicum* transmission seemed attainable by introduction of effective programs of chemotherapy (using Praziquantel) to eliminate adult worms in the human or livestock hosts, and proper sanita-

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tion concurrent together with the indiscriminant application of molluscicides to exterminate the snails. Although such integrated programs have had dramatic success in some areas of China (Jiang et al., 2002), the sustained effort required for their maintenance in contiguous water bodies requires considerably more resources than are available in many affected areas (World Health Organization, 1993). Additionally, synthetic molluscicides can cause serious environmental damage to freshwater ecosystems. In China, water development schemes, such as the Three Gorges Dam on the Yangtze River and the south-to-north water transfer project, may encourage growth and spread of *Oncomelania hupensis*, creating a need for further basic malacological research.

The subspecies and populations of *Oncomelania hupensis* differ in shell size and structure, breeding environment, growth rates, population genetics, and potential for infection by *S. japonicum* (Yuan et al., 1984; Hope & McManus, 1994; Davis et al., 1995, 1999a, 2006; Wilke et al., 2000; Zhou et al., 2005b). Based on congruence of allozymes and mitochondrial cytochrome oxidase I [COI] gene sequences (Davis et al., 1995, 1999a, b; Wilke et al., 2000; Shi et al., 2002), there are three distinct clades of *Oncomelania hupensis* in the mainland China, widely separated biogeographically. These have been assigned to three subspecies. *Oncomelania h. hupensis* thrives throughout the Yangtze River drainage system below the Three Gorges to Shanghai, Jiangsu, and Zhejiang provinces (some populations are dispersed at high altitudes). This subspecies has populations with smooth or ribbed shells (Davis et al., 2006). *Oncomelania h. robertsoni* is located in Sichuan and Yunnan provinces at high elevations (500 m, most 1,000 m above sea level) above the Three Gorges of the Yangtze River and has the ancestral (pleisiomorphic) small, smooth shell without a varix (thickening of the outer lip of the shell) (Davis, 1979; Davis et al., 1995); *O. h. tangi* is found in Fujian Province along the southern coast of China, opposite Taiwan, separated from the Yangtze River by high mountain ranges. The shells are smooth but have a doubly thick varix, and their allometry differs from that of the other two subspecies in that their shells are proportionally much wider for their length.

As was shown by previous allozyme studies, the introduction of new molecular-based methodologies has the potential to greatly improve differentiating populations and higher taxa. In recent years, some universal PCR primers were used to amplify targets in nuclear and mitochondrial genomes across a broad range of taxa (Simon et al., 1994; Pedersen, 1996), and the introduction of arbitrary primer mediated DNA fingerprinting (Williams et al., 1990; Welsh & McClelland, 1990; Caetano-Anolles et al., 1991; Vos et al., 1995). The choice of these universal primer methods for species identification and elucidation of relationships in tropical freshwater snails was discussed by Jones et al. (1999). The Randomly Amplified Polymorphic DNAs (RAPDs) method was successfully utilized for species identification and clarification of genetic relationships among planorbid snails in Central and East Africa (Jones et al., 1999). In contrast, Amplified Fragment Length polymorphisms (AFLPs) combine RAPDs and RFLPs to assay anonymous sequences over the entire genome, but with the major advantage of being more robust and reliable than other arbitrary primer-mediated DNA fingerprinting methods, because amplification is performed under more stringent reaction conditions (Vos et al., 1995). The AFLP markers have been used to infer phylogenetic relationships of closely related species based on measures of genetic distance (Heun et al., 1997; Semblat et al., 1998). Based on analyses mtDNA, LSU rRNA, and AFLPs of *O. hupensis robertsoni* specimens, Wilke et al. (2006) found that there was no clear phylogenetic concordance between the mitochondrial and nuclear data in *Oncomelania hupensis robertsoni* (in contrast with *O. h. hupensis*). They strongly recommended that future studies incorporate more data from nuclear loci in order to better understand phylogeography, population genetics, and host-parasite coevolution.

The purposes of this paper are (1) to use AFLP methodology to assess the degree to which AFLPs derived relationships are congruent with the allozymes and COI results; (2) to determine the degree to which AFLPs data enables analysis of population structure.

MATERIALS AND METHODS

Locality Data

Oncomelania is distributed in twelve provinces of mainland China. We collected samples of 25 snail populations from ten provinces. We did not find sufficient samples for studying in Shanghai and Guangdong prov-

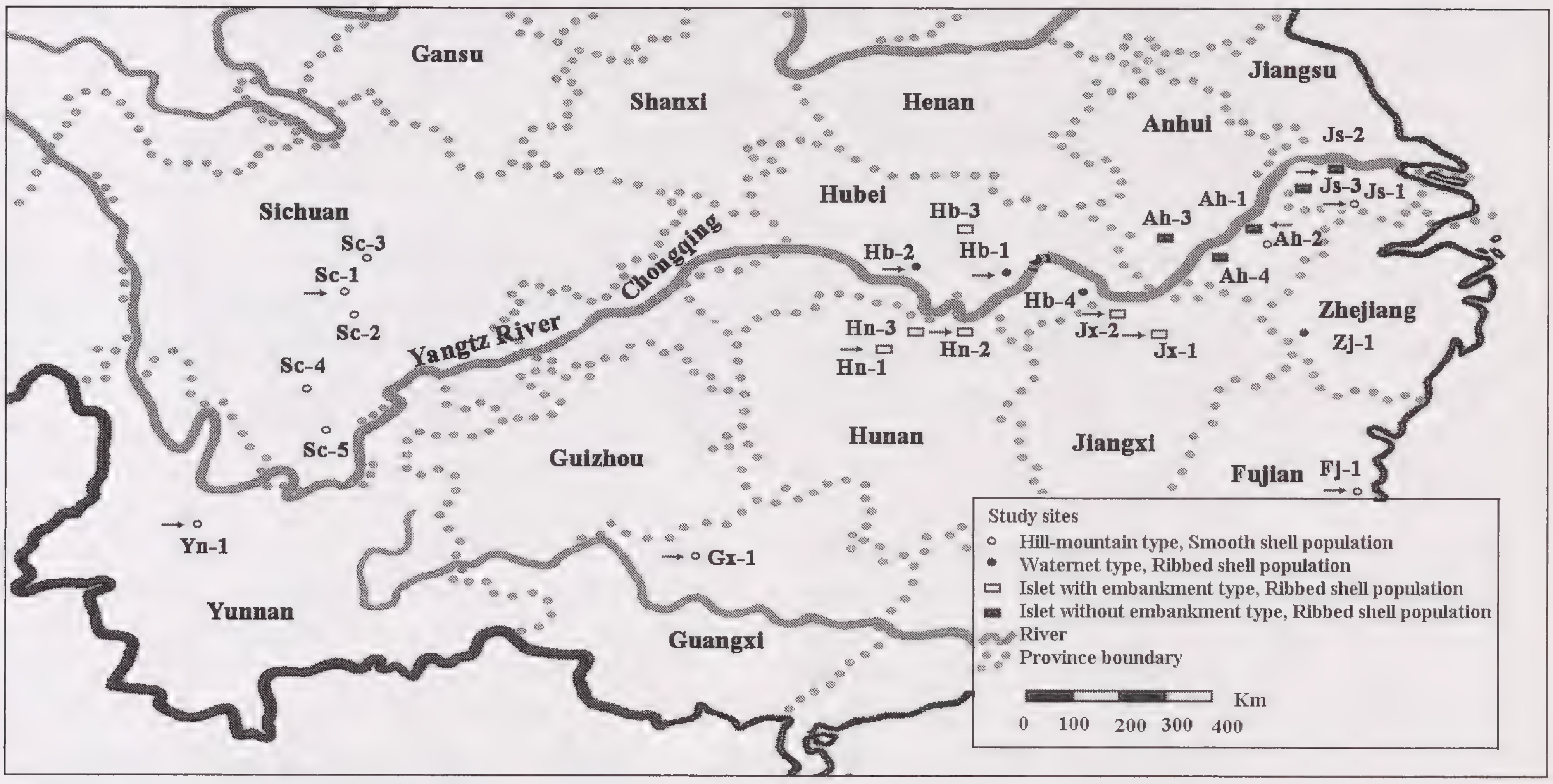

FIG. 1. Localities studied. Populations pointed out by arrows were used for studies of intra-population variation.

inces. A total of 760 uninfected individuals from 25 *O. hupensis* populations were studied (Fig.1). Analyses were done on 40 individuals randomly sampled from the *O. hupensis* population from Hn-2; 30 individuals were analyzed from each of the other populations. Locality information is given in Table 1.

Snail Selection

Individuals (120) were collected at random from each *O. hupensis* population. The snails were isolated without food, in an antibiotic solution (ampicillin 100 µg/ml) and left overnight at 4°C to minimize prokaryotic growth prior to DNA extraction. Then the cercaria shedding test was used to examine whether they were infected with *S. japonicum*. Those positive snail individuals were eliminated. The remaining individuals were crushed and examined microscopically for larval trematodes, then the negative individuals were selected for DNA isolation.

DNA Extraction

Genomic DNA was extracted from the foot of each snail individual using a modified CTAB protocol (Enjalbert et al., 2002). The DNA concentrations were determined by O.D. measurement using a BioPhotometer (Eppendorf, Hamburg, Germany). All samples were then diluted to a concentration of 50ng/µl. After dilution, additional measurements were performed to ensure the correct concentration.

Population Used to Study Variation with Increased Sample Size and Number of AFLP Molecular Loci

The Hn-2 population was selected to analyze how much variation increasing with sample size and the number of AFLP molecular loci.

Populations Used to Study Intra-Population Genetic Variation

Thirteen snail populations (Yn-1, Js-1, Sc-1, Fj-1, Gx-1, Js-2, Ah-1, Jx-1, Jx-2, Hn-1, Hn-2, Hb-1, Hb-2) were selected from the 25 snail populations to determine the amount of intra-population variation. Of these snail populations, ten populations (Hn-1, Hn-2, Js-1, Js-2, Gx-1, Hb-1, Hb-2, Ah-1, Jx-1 and Jx-2) from six provinces belong to *O. h. hupensis*; two populations (Yn-1 and Sc-1) are *O. h. robertsoni*; one population (Fj-1) is *O. h. tangi*.

DNA Pool Construction

For each snail population, 30 µl from 30 snail individuals (1 µl per individual) from the same population were pooled together to assess the molecular genetics of *O. hupensis*.

TABLE 1. Locality information for twenty five snail populations of *Oncomelania hupensis* studied.

Locality code	Shell sculpture	Varix	Locality	Latitude	Longitude
Hn-1	Ribbed	Yes	Hunan, Nanxian County, Babaigong Township, Xinshang Village	29.1365°N	112.3329°E
Hn-2	Ribbed	Yes	Hunan,Yueyang City, Junshan District, Liulinzhou Township, Changjiang Village	29.3881°N	112.8595°E
Hn-3	Ribbed	Yes	Hunan, Hua Rong County, Xing Fu Township, Dong Jia Village	29.3060°N	112.7202°E
Yn-1	Smooth	No	Yunnan, Dali City, Yinqiao Township, Wuliqiao Village	25.6686°N	100.1789°E
Js-1	Smooth	Yes	Jiangsu, Yixing City, Hufu Township, Zhanggong Village	31.2254°N	119.8871°E
Js-2	Ribbed	Yes	Jiangsu, Dantu County, Shiye Township, Xintan Village	32.2107°N	119.2855°E
Js-3	Ribbed	Yes	Jiangsu, Jiang Ning District, Tong Jing Township, Xin Ji Zhou Village	31.7932°N	118.5447°E
Sc-1	Smooth	No	Sichuan, Pujiang County, Changqiu Township, Sanhe Village	30.2167°N	103.6370°E
Sc-2	Smooth	No	Sichuan, Dan Leng County, Yang Chang Township, Xu Ba Village	29.9131°N	103.5502°E
Sc-3	Smooth	No	Sichuan, Guang Han City, Shuang Quan Township, Long Quan Village	30.9952°N	104.4267°E
Sc-4	Smooth	No	Sichuan, Xi Chang City, Chuan Xing Township, Xin Long Village	27.8670°N	102.3237°E
Sc-5	Smooth	No	Sichuan, Pu Ge County, Te Zi Township, Chang Shou Village	27.6129°N	102.6653°E
Fj-1	Smooth	Yes	Fujian, Fuqing City, Donghan Township, Donghan Village	25.4130°N	119.6140°E
Gx-1	Smooth	Yes	Guangxi, Yizhou City, Fulong Township, Jingkou Village	24.5009°N	108.1668°E
Hb-1	Ribbed	Yes	Hubei, Wuhan City, Caidian District, Xiaosi Township, Wagou Village	30.4471°N	113.6889°E
Hb-2	Ribbed	Yes	Hubei, Jiangling County, Zishi Township, Fanyuan Village	30.1842°N	112.4084°E
Hb-3	Ribbed	Yes	Hubei, Han Chuan City, Liu Ge Township, Xi Si Tai Village	30.7575°N	113.8114°E
Hb-4	Ribbed	Yes	Hubei, Yang Xin County, Mu Gang Township, Xin Hu Village	29.7265°N	115.2476°E
Ah-1	Ribbed	Yes	Anhui, Tongling County, Laozhou Township, Guanghui Village	30.9723°N	117.7477°E
Ah-2	Smooth	Yes	Anhui, Tong Ling County, Shun An Township, Chang Shan Village	30.9538°N	117.9407°E
Ah-3	Ribbed	Yes	Anhui, Cong Yang County, Feng Yi Township, Feng Yi Village	30.7106°N	117.4407°E
Ah-4	Ribbed	Yes	Anhui, Gui Chi District, Mei Long Township, Guo Gang Village	30.7673°N	117.6423°E
Jx-1	Ribbed	Yes	Jiangxi, Douchang County, Zhouxi Township, Chaipeng Village	29.1822°N	116.3912°E
Jx-2	Ribbed	Yes	Jiangxi, Xingzi County, Xinchi Township, Zhuxi Village	29.3098°N	116.0047°E
Zj-1	Ribbed	Yes	Zhejiang, Jiangshan County, Wangyao Township, Dahe Village	28.6890°N	118.6597°E

Amplified Fragment Length Polymorphism (AFLP) Reaction

AFLP protocol was adapted from Mikkonen et al. (2005) with some modifications as follows: two hundred nanograms of genomic DNA was digested with 8 units of each restriction Enzyme *PstI* and *MseI* (New England Biolabs), and then restriction fragments were ligated to the *PstI* adapter and *MseI* adapter at 37°C for 5 h in a restriction-ligation buffer [2.5µl 10 × Reaction buffer, 1 µl *PstI* / *MseI* adapter, 2 µl *PstI* / *MseI*, 2.5 µl 10mM ATP, 3 unit of T4 DNA Ligase, 7 µl Ultrapure Water]. The final volume of the mixture was 20 µl. After a 1 : 20 dilution in ultrapure water, ligation products were used directly (no streptavidine purification) for the pre-amplification, using *Pst* and *Mse* primers (2 ng/µL) with no selective nucleotides.

In the pre-amplification, 2 µl of ligated restriction fragments was used as template. 1 µl unlabelled *PstI* -0 primer and *MseI* -0 primer (MWG-Biotech AG) were added to a amplification buffer [1 µl dNTPs, 2.5 µl 10Reaction buffer, 1 unit of T4 DNA Ligase, 18 µl Ultrapure Water], giving a total volume of 25 µl. Preselective amplification was performed at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 80 s and at last 72°C for 5 min.

In the selective amplification, the products of the pre-amplification were diluted 1 : 20 in ultrapure water, and followed by a specific amplification with three selective nucleotides on both *MseI* and *PstI* primers (Table 2). Selective DNA amplification was performed in a 20 µl reaction mixture consisting of 2 µl of template DNA from the preselective PCR, 2 µl 10x Reaction buffer, 1 µl dNTP, 1 µl fluorescence labeled *PstI* primer (5 ng/µl), 1 µl fluorescence labeled *MseI* primer (30 ng/µl), 1 unit of T4 DNA Ligase, and 12.5 µl Ultrapure Water. Selective amplification was done by incubating at 94°C for 2 min, then one cycle of 94°C for 30 s, 65°C for 30 s and 72°C for 80 s. The annealing temperature was then reduced by 0.7°C in each subsequent cycle until attaining 56.6°C (12 cycles), followed by an extra 23 cycles at a 94°C for 30 s, 55°C for 30, 72°C for 80 s, and at last 72°C for 5 min.

PCR products were separated electrophoretically using a 6% polyacrylamide gel and an automatic ABI 377 DNA Sequencer (GMI, Inc.). The resulting band patterns were analyzed visually. AFLP fragments of strong intensity were scored as binary characters for each isolate.

Data Analysis

The AFLP markers, treated as alleles, were analyzed following the approach used for RAPD (Random Amplified Polymorphic DNA) markers (Luch, 1990; Vekemans et al., 2002). The percentage of polymorphic loci, Nei's gene diversity (Nei, 1973), and Shannon's information index (Lewontin et al., 1972) were computed using the software package PopGen32 (version 1.32) (Yeh, 1999). Pairwise similarities (GS_{Dice}) were calculated between snail populations using Dice's coefficient (Nei & Li, 1979) by Simqual program of the software packages NTSYpc (Rohlf, 1998), and the similarity coefficients (GS_{Dice}) were converted to genetic distance (Genetic distance D = 1-GS_{Dice}).The

TABLE 2. 12 primer combinations used for AFLP analysis, and their sequences.

Primer Pair	*PstI*	Primer Sequence	*MseI*	Primer Sequence
Pre-	P0	5'-GACTGCGTACATGCA-3'	M0	5'-GATGAGTCCTGAGTAA-3'
A7	P1	5'-GACTGCGTACATGCAGAA-3'	M7	5'-GATGAGTCCTGAGTAACTG-3'
A8	P1	5'-GACTGCGTACATGCAGAA-3'	M8	5'-GATGAGTCCTGAGTAACTT-3'
B4	P2	5'-GACTGCGTACATGCAGAC-3'	M4	5'-GATGAGTCCTGAGTAACAT-3'
B6	P2	5'-GACTGCGTACATGCAGAC-3'	M6	5'-GATGAGTCCTGAGTAACTC-3'
B7	P2	5'-GACTGCGTACATGCAGAC-3'	M7	5'-GATGAGTCCTGAGTAACTG-3'
B8	P2	5'-GACTGCGTACATGCAGAC-3'	M8	5'-GATGAGTCCTGAGTAACTT-3'
F5	P6	5'-GACTGCGTACATGCAGTC-3'	M5	5'-GATGAGTCCTGAGTAACTA-3'
F7	P6	5'-GACTGCGTACATGCAGTC-3'	M7	5'-GATGAGTCCTGAGTAACTG-3'
G5	P7	5'-GACTGCGTACATGCAGTG-3'	M5	5'-GATGAGTCCTGAGTAACTA-3'
G7	P7	5'-GACTGCGTACATGCAGTG-3'	M7	5'-GATGAGTCCTGAGTAACTG-3'
G8	P7	5'-GACTGCGTACATGCAGTG-3'	M8	5'-GATGAGTCCTGAGTAACTT-3'
H8	P8	5'-GACTGCGTACATGCAGTT-3'	M8	5'-GATGAGTCCTGAGTAACTT-3'

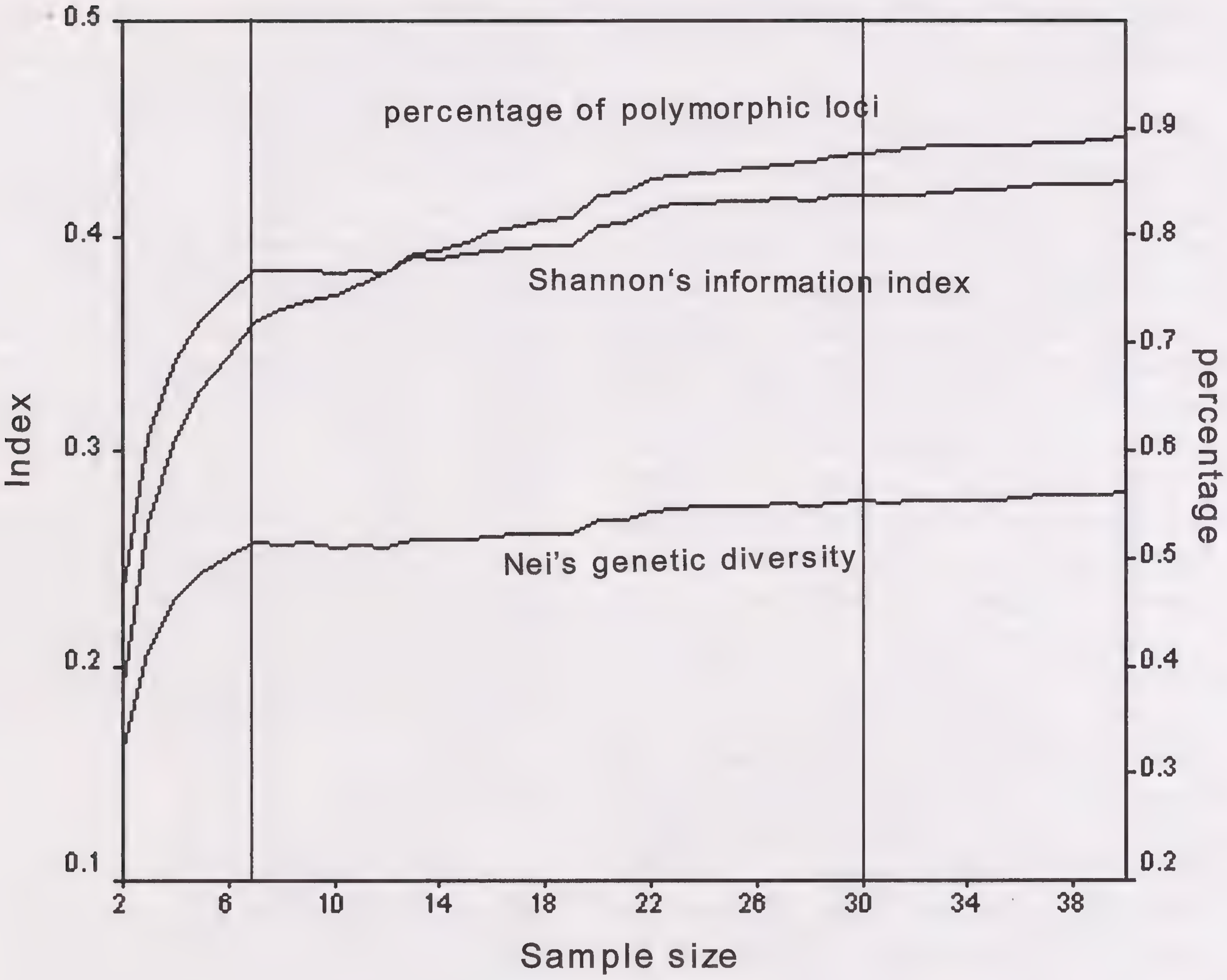

FIG. 2. Information on intra-population genetic diversity with snail sample size increasing.

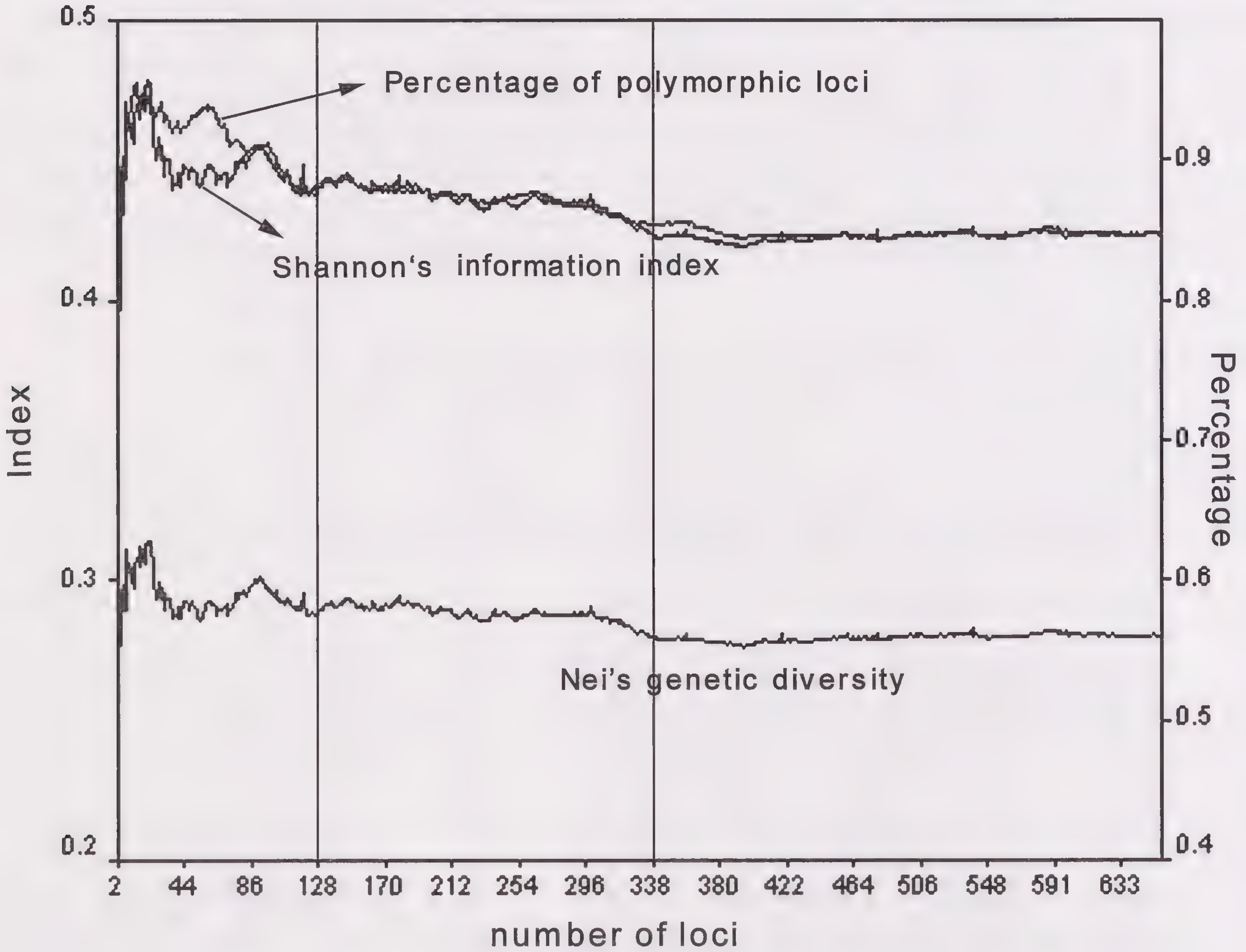

FIG. 3. Information on intra-population genetic diversity with the number of AFLP loci increasing.

genetic diversities were compared among multiple snail populations with Kruskal-Wallis H nonparametric test, between subspecies of *O. hupensis* with Mann-Whitney U of the nonparametric test. GS_{Dice} were compared between subspecies of *O. hupensis* with bootstrap approach. The other data analysis and graphs drawing were performed using the software packages Microsoft Excel (Microsoft Corporation, USA) and SPSS (SPSS Inc., Chicago, USA).

RESULTS

Relationship between Stability of Information on Genetic Variation and Sample Size

The AFLP method was used to analyze the 40 individuals of *O. hupensis* from Hn-2 with 12 primer pairs. 42–74 distinct fragments with a size in the range of 50–490 bp were yielded for each primer combination, and the percentage of polymorphic loci was approximately 82.9%–93.9%. Overall, the percentage of AFLP polymorphic loci, Nei's gene diversity, and Shannon's information index increased gradually with increasing sample size. There were three phases: (a) The rapidly changing phase: when sample size increased from 2 to 7 snail individuals, the three indices above increased rapidly. (b) The slowly changing phase: when sample size increased from 7 to 30 individuals, the values of the three indices increased slowly. (c) The stable phase: when sample size was more than 30 snails, the values of these indices trended to stabilization (Fig. 2). The snail individuals in the order on the x-axis were randomized.

Relationship Between Stability of Information on Genetic Variation and Number of AFLP Molecular Loci

Thirty out of 40 snails from Hn-2 above were selected at random to analyze how much variation increased with the number of AFLP loci. With the increase in the number of AFLP loci, the change of the percentage of polymorphic loci and the indices of genetic diversity was divided approximately into three phases as above: (a) The phase of rapid change: when the number of AFLP loci increased from 2 to 128, the values of the percentage of polymorphic loci and the indices of genetic diversity fluctuated rapidly. (b) The phase of slow change: when it increased from 128 to 338, the values of these indices fluctuated slowly. (c) The stable phase: when the number of AFLP loci was more than 338, the values of these indices trended to stabilization (Fig. 3). The loci in the order on the x-axis were randomized.

Intra-Population Genetic Diversity

Thirteen populations (Fig. 1) were studied by AFLP method with eight primer pairs (namely A7, A8, B4, B6, B7, G5, G7, and G8). Within single locality population, the number of fragments amplified by AFLP method ranged from 403 to 472 with the number of

TABLE 3. Genetic diversities within 13 populations of *Oncomelania hupensis*.

Population code	No. loci	No. of polymorphic loci	Percentage of polymorphic loci (%)	Nei's gene diversity	Shannon's information index
Yn-1	436	379	86.87	0.322 ± 0.0026	0.476 ± 0.0035
Js-1	403	314	77.92	0.270 ± 0.0030	0.404 ± 0.0041
Sc-1	424	314	74.03	0.285 ± 0.0031	0.421 ± 0.0043
Fj-1	430	304	70.61	0.253 ± 0.0029	0.377 ± 0.0042
Gx-1	405	226	55.80	0.191 ± 0.0031	0.287 ± 0.0045
Js-2	467	418	89.51	0.333 ± 0.0023	0.493 ± 0.0031
Ah-1	426	370	86.85	0.322 ± 0.0025	0.478 ± 0.0035
Jx-1	434	378	87.10	0.313 ± 0.0026	0.465 ± 0.0035
Jx-2	472	440	93.22	0.345 ± 0.0022	0.510 ± 0.0029
Hn-1	459	403	87.77	0.339 ± 0.0025	0.500 ± 0.0034
Hn-2	453	391	86.31	0.276 ± 0.0027	0.420 ± 0.0037
Hb-1	433	371	85.88	0.330 ± 0.0026	0.485 ± 0.0035
Hb-2	415	343	82.55	0.302 ± 0.0029	0.450 ± 0.0040

polymorphic loci from 226 to 440; a percentage of polymorphic loci ranged from 55.80% to 93.22%, and the Nei's gene diversity from 0.191 to 0.345; the Shannon's information index ranged from 0.287 to 0.510. The genetic diversities within *Oncomelania* populations from different areas differed significantly (Nei's gene diversity $\chi^2 = 2500.56$, $P < 0.01$; Shannon's information index $\chi^2 = 2501.20$, $P < 0.01$). Of thirteen snail populations, the genetic diversity within Gx-1 was the lowest, and the percentage of polymorphic loci, Nei's gene diversity, Shannon's information index and average coefficient of similarity were 55.80%, 0.191, 0.287 and 0.904 respectively; whereas the percentage of polymorphic loci, Nei's gene diversity and Shannon's information index of Jx-2 were the most and were 93.22%, 0.345 and 0.510 respectively (Table 3).

Inter-Population Genetic Variation

DNA pools of 25 five snail populations were amplified using the AFLP method with eight primer pairs (A7, A8, B4, B6, B7, G5, G7, and G8). These distinct amplified fragments with a size in the range of 50 to 325 bp were selected to analyze the genetic variation among snail populations.

Among 25 snail populations, Nei's gene diversity and Shannon's information index were 0.280 and 0.426 respectively; genetic distance D from 0.169 to 0.306, and Nei's unbiased genetic distance from 0.225 to 0.452. Among six snail populations of *O. h. robertsoni*, genetic distance D was from 0.170 to 0.235, and Nei's unbiased genetic distance was from 0.225 to 0.334. Among 18 snail populations of *O. h. hupensis*, genetic distance D was in the range of 0.176 to 0.289, and Nei's unbiased genetic distance was in the range of 0.229 to 0.413. Whether Nei's gene diversity, Shannon's information index, genetic distance D, or Nei's unbiased genetic distance, among-snail population distance of *O. h. hupensis* was greater than that between *O. h. robertsnoi* populations ($P < 0.01$). Of 18 snail populations of *O. h. hupensis,* 15 snail populations had ribbed-shells, and three snail populations had smooth shells. Nei's gene diversity and Shannon's information index among ribbed-shell populations of *O. h. hupensis* were higher than that among smooth-shelled populations of *O. h. hupensis* ($P < 0.01$), but genetic distance D and Nei's unbiased genetic distance among ribbed-shelled populations of *O. h. hupensis* were lower than that among smooth-shelled populations of *O. h. hupensis* ($P < 0.01$). If the smooth-shelled populations of *O. h. hupensis* were not included a snail population from Guangxi province, Genetic distance D and Nei's unbiased genetic distance among smooth-shelled populations of *O. h. hupensis* were not different from that among ribbed-shelled populations of *O. h. hupensis* ($P > 0.05$). Nei's gene diversity and Shannon's information index among populations of *O. h. robertsoni* were not different from that among smooth-shell populations of *O. h. hupensis* ($P > 0.05$), but genetic distance D and Nei's unbiased genetic distance among smooth-shell populations of *O. h. hupensis* were higher than

TABLE 4. Genetic variation among the populations of *Oncomelania hupensis*.

	O. h. robertsoni	*O. h. hupensis*			Total
		Ribbed	Smooth	Total	
Nei's genetic diversity	0.201 ± 0.0030	0.253 ± 0.0028	0.209 ± 0.0035	0.262 ± 0.0027	0.280 ± 0.0026
Shannon's information index	0.300 ± 0.0034	0.384 ± 0.0039	0.300 ± 0.0051	0.399 ± 0.0037	0.426 ± 0.0035
Nei's unbiased genetic distance	0.278 ± 0.0088	0.310 ± 0.0035	0.380 ± 0.0150	0.328 ± 0.0032	0.328 ± 0.0039
Nei's unbiased genetic identity	0.758 ± 0.0067	0.729 ± 0.0026	0.684 ± 0.0101	0.722 ± 0.0023	0.722 ± 0.0028
GS_{Dice}	0.797 ± 0.0055	0.765 ± 0.0022	0.722 ± 0.0064	0.759 ± 0.0020	0.761 ± 0.0024
Genetic distance D	0.203 ± 0.0055	0.235 ± 0.0022	0.278 ± 0.0064	0.241 ± 0.0020	0.239 ± 0.0024

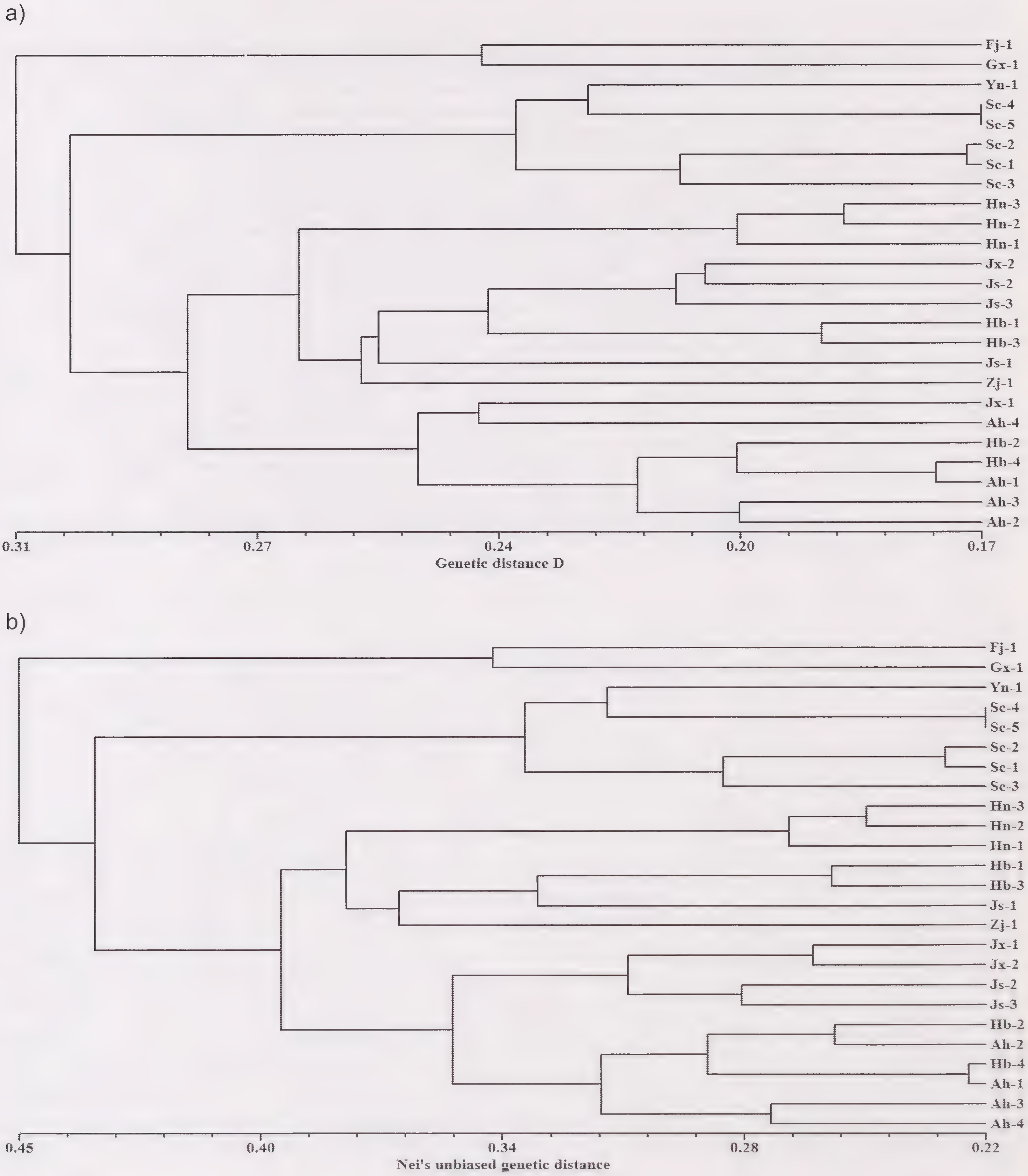

FIG. 4. Complete-link trees depicting phylogenetic relationships among twenty five snail populations of *O. hupensis* using (a) Genetic distance D matrix and (b) Nei's unbiased genetic distance matrix.

that among populations of *O. h. robertsoni* ($P < 0.01$). Whether Nei's gene diversity, Shannon's information index, Genetic distance D or Nei's unbiased genetic distance among ribbed-shell populations of *O. h. hupensis* was larger than that among snail populations of *O. h. robertsoni* ($P < 0.01$) (Table 4).

Snail AFLP Phylogeny

Analyses of Genetic distance D and Nei's unbiased genetic distance matrixes using the Complete-link method, Neighbor-joining (NJ) method, Single-link method and Unweighted pair-group method with arithmetic means

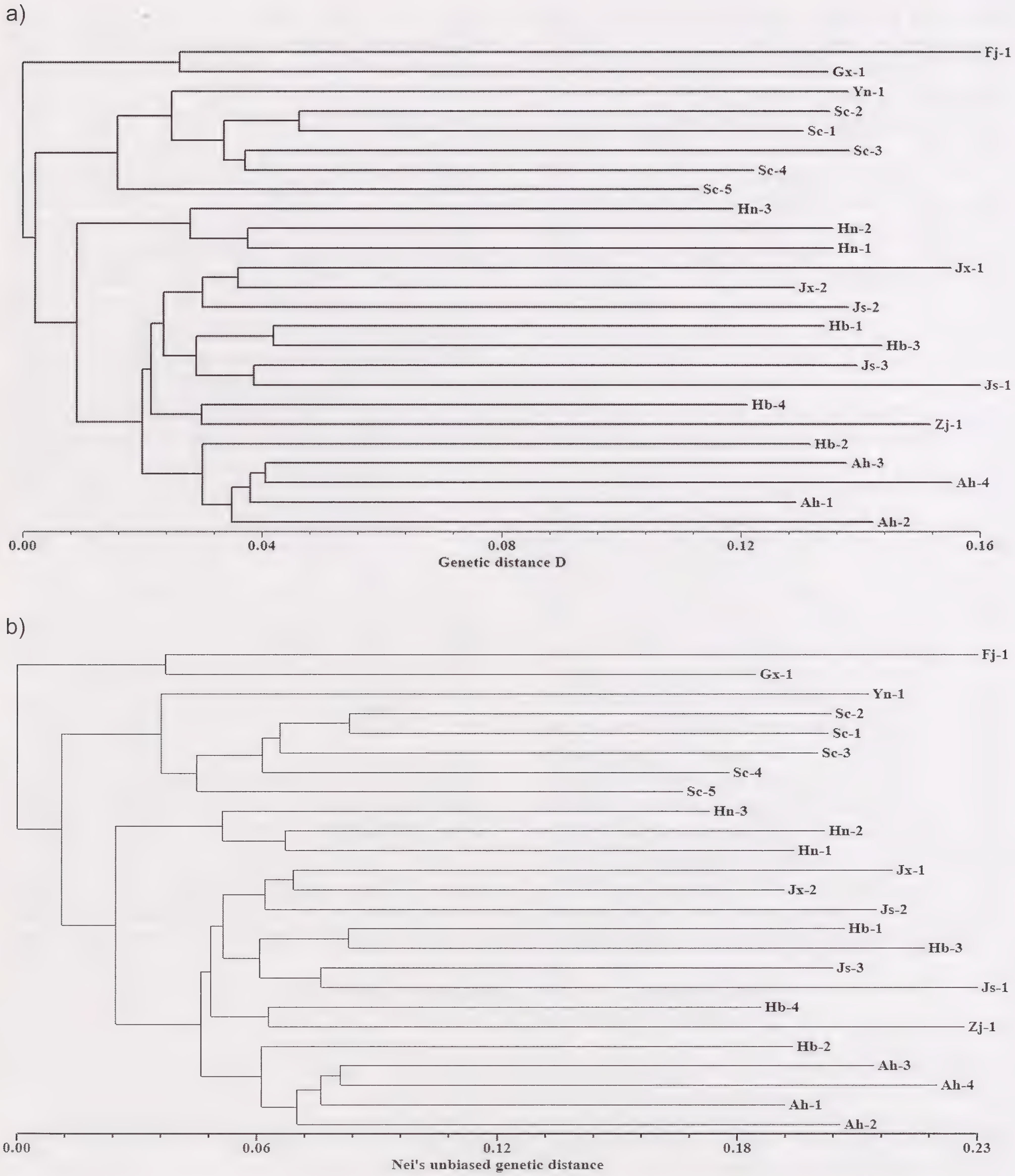

FIG. 5. Neighbor-joining trees depicting phylogenetic relationships among twenty five snail populations of *O. hupensis* using (a) Genetic distance D matrix and (b) Nei's unbiased genetic distance matrix.

(UPGMA) method respectively were shown in Figs. 4–7. For the complete-link method and NJ method, whether Genetic distance D matrix or Nei's unbiased genetic distance matrix was analyzed, the two Complete-link trees and the two NJ trees were similar to each other, and especially the NJ phylogram using Genetic distance D matrix was very similar to the NJ tree using Nei's unbiased genetic distance matrix. These four trees were all rooted with Fj-1 and Gx-1 populations and showed two big branches. One branch included six smooth-shelled populations without varix (Yn-1, Sc-1-Sc-5) from Yunnan and Sichuan Prov-

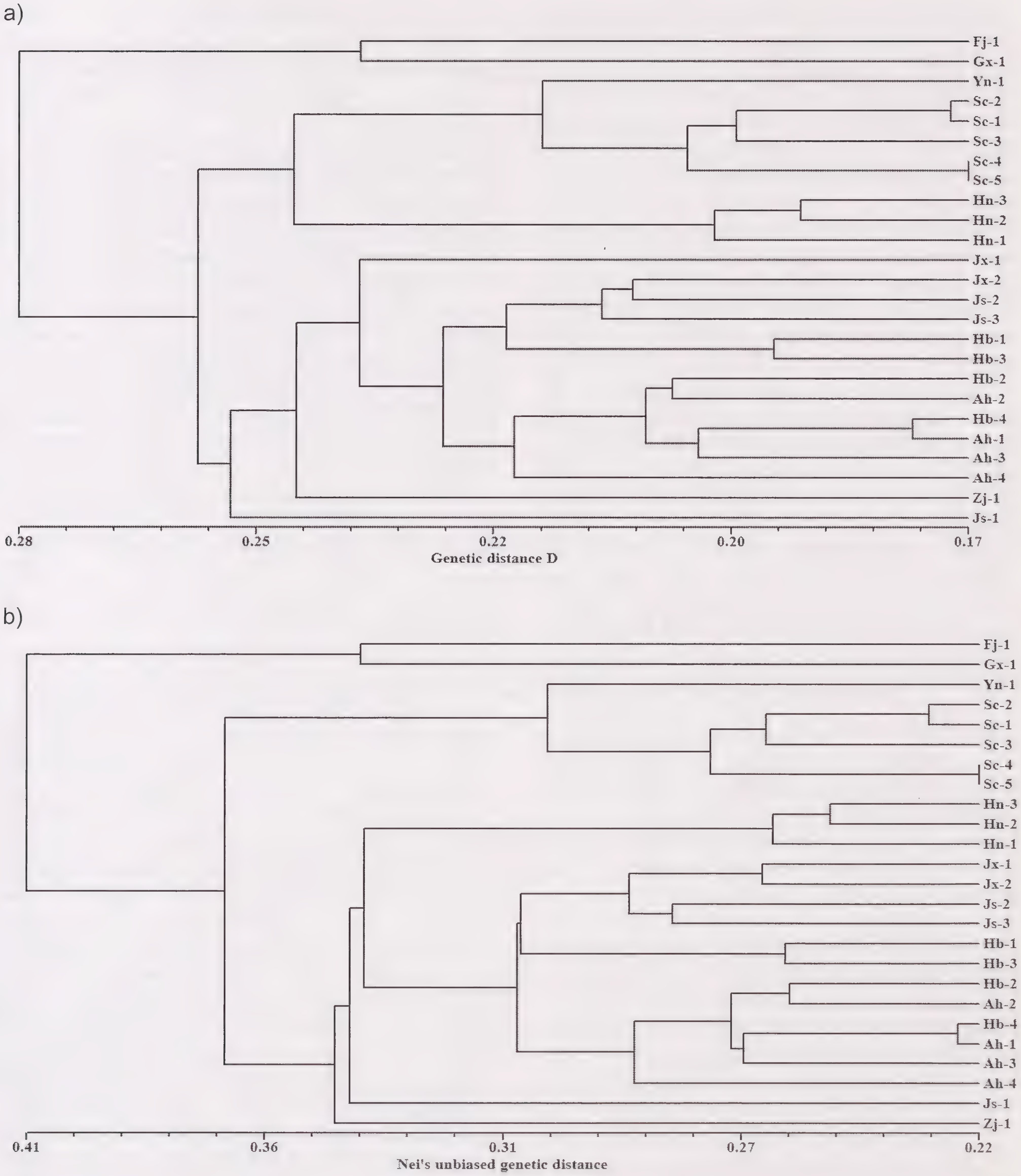

FIG. 6. UPGMA trees depicting phylogenetic relationships among twenty five snail populations of *O. hupensis* using (a) Genetic distance D matrix and (b) Nei's unbiased genetic distance matrix.

inces, the other branch consisted of other 17 snail populations from throughout the Yangtze River drainage below the Three Gorges to Jiangsu and Zhejiang provinces. The two UPGMA trees also were both similar to the four trees above, and were both rooted with Fj-1 and Gx-1 populations and showed two big branches. Unexpectedly, three ribbed-shelled populations (Hn-1, Hn-2 and Hn-3) from Hunan Province fell within the *O. hupensis robertsoni* subspecies (which is found in Yunnan and Sichuan provinces) clade in the UPGMA tree using Genetic distance D matrix, but the genetic distance D between

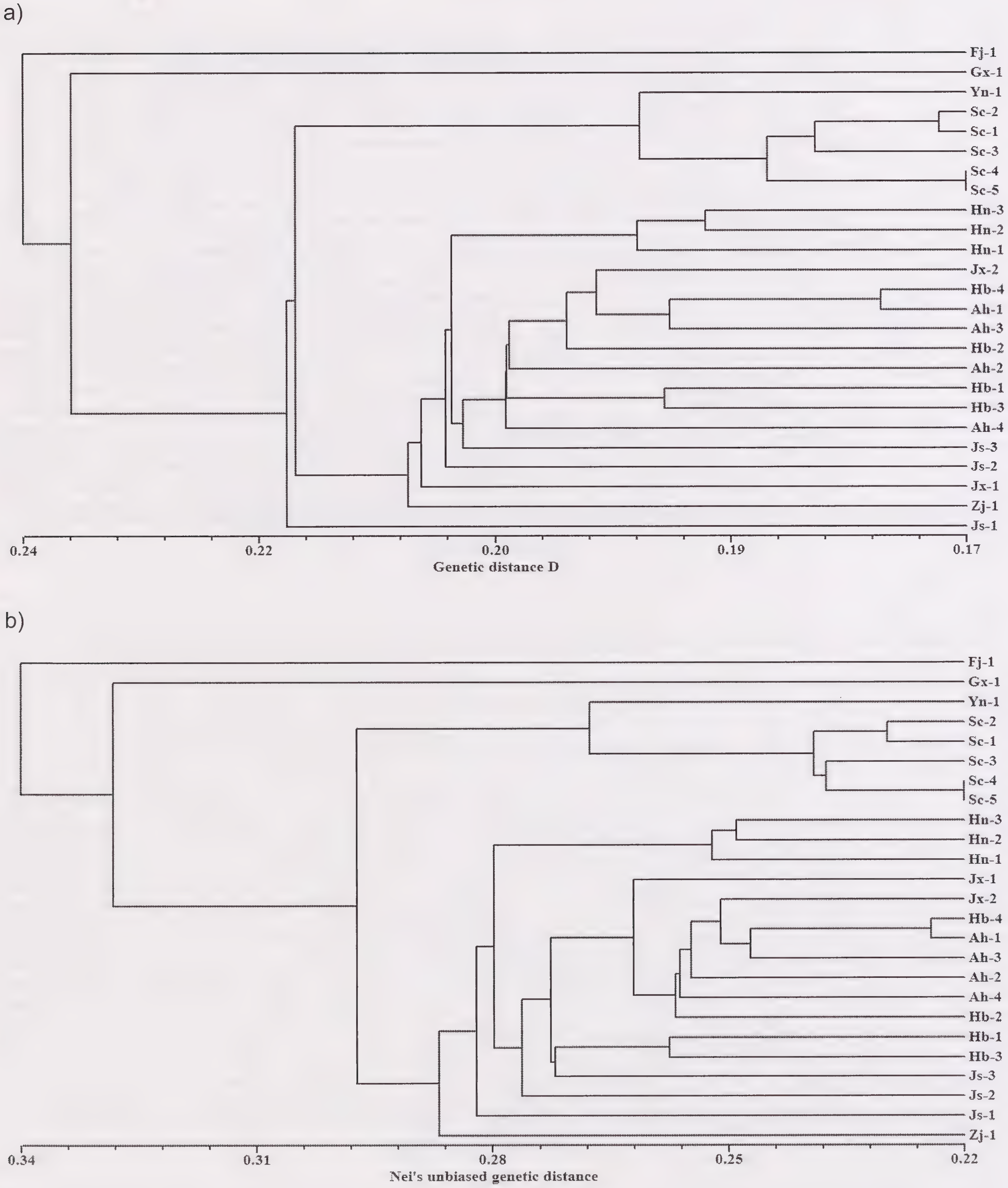

FIG. 7. Single-link trees depicting phylogenetic relationships among twenty five snail populations of *O. hupensis* using (a) Genetic distance D matrix and (b) Nei's unbiased genetic distance matrix.

the snail populations from Hunan Province and the snail populations from Yunnan and Sichuan provinces was relatively big (0.220 to 0.298). The two single-link trees were different from six other trees above, and were both rooted with only Fj-1 populations, with Gx-1 population divided into an independent branch. The other 23 snail populations also were divided similarly into two big branches except the Js-1 population in the tree using genetic distance D matrix, which was divided into an independent branch.

DISCUSSION

Appropriate Sample Size and Number of AFLP Markers

Due to intra-population genetic variation, one or several snail individuals are insufficient to analyze population variation. The snail population (Hn-2) selected to examine how much variation increased with sample size, and the number of AFLP molecular loci showed that the percentage of polymorphic loci, Nei's gene diversity, and Shannon's information index all increased gradually with increasing sample size up to a sample size of 30 or more individuals, When the sample size was less than seven individuals, the values of these indices are very unstable. Our result is consistent with the sample size calculated based on the formula of Brown & Moran (1981) in a random mating population. So we sampled randomly 30 snail individuals not infected with *S. japonicum* from a snail population to represent this population in this paper. We recognize the fact that if additional populations were added, variation would continue to increase. The practical solution, universally recognized, is to use between 15 to 25 individuals for population genetic studies.

Our AFLP analysis data still showed, on basis of 30 snails sample from Hn-2 population, that the percentage of polymorphic loci, Nei's gene diversity, and Shannon's information index change rather acutely when the number of loci is less than 128, while the values of theses indices trend to stabilization when the number of loci is more than 338. These indicate that the appropriate number of AFLP markers for studying the genetic variations of *O. h.* by AFLP method is optimally not less than 338 for each *Oncomelania* population, whereas the result is very unreliable when the number of AFLP markers is less than 128. In our study, the total eight primer pairs were used to analyze genetic diversity of *O. hupensis*, and the number of AFLP markers amplified was in range of 403–467 bands for the 13 *Oncomelania* populations. So it was enough and reasonable to use only eight primer pairs for studying and comparing the genetic variation of difference *Oncomelania* populations.

Intra-Population Genetic Diversity

In a previous allozyme study of *O. hupensis*, Davis et al. (1995) found that 14 snail populations from Mainland China had few alleles per locus (1.0 to 1.6), a low mean heterozygosity (0.008 to 0.036), and low percentage polymorphic loci (4 to 28). In another previous COI gene sequencing study of *O. hupensis*, Wilke et al. (2000) also found relatively little genetic variability within the in-group individuals, and the nucleotide diversity ranged from 0 to 0.0196. Compared with these relatively conservative allozyme and COI marker, our AFLP data showed that there was higher intra-population genetic variation in mainland China – the percentage of polymorphic loci ranged from 55.80% to 93.22%, the Nei's gene diversity from 0.191 to 0.345, and the Shannon's information index from 0.287 to 0.510 – furthermore, these intra-population genetic variations from different areas differed significantly. These substantial differences were present among not only the three subspecies, but also different populations of the same subspecies, especially *O. h. hupensis.* The results found in our study were similar to those by Wilke et al. (2006), and they found each snail individual had a unique AFLP fingerprint in the preliminary AFLP analysis of 24 specimens. So our results might indicate that AFLP approach can examine more genetic diversity of *O. hupensis* than allozyme and COI gene sequence.

Oncomelania hupensis tangi has been eradicated except for two known populations, and the parasite presumably is extinct. Our AFLP data showed interestingly that the genetic variation within Fj-1 from *O. h. tangi* as well as Gx-1 population was very low. *Schistosomiasis japonicum* in Guangxi Province has been exterminated, except for intermediate snail hosts restricted in narrow areas. This might be mostly due to the fact that these snail populations are localized relatively narrow areas, and the number of individuals in these snail populations is very limited, so the genetic drift can easily happen in these snail populations, and the results of the genetic drift will lead to farther decrease of their genetic diversity.

Inter-Population Genetic Diversity and Phylogeny

Our AFLP analysis data from 25 snail populations show that considerable genetic differentiation has been occurred throughout China. Genetic distance D was from 0.169 to 0.306, and Nei's unbiased genetic distance from 0.225 to 0.452. These two indices of genetic distance with the four clustering methods were used to reconstruct the phylograms of *O. hupensis* from mainland China, and most results of the eight phylogenetic trees were similar, and patterns of genetic differentiation were in general correlated with geographic distribution. These results were similar to the results of allozymes and COI gene sequences (Davis et al., 1995, 1999a, b; Wilke et al., 2000; Shi et al., 2002) excluding a snail population from Guangxi Province (Gx-1). These patterns thus suggested that there were three distinct clades of *O. hupensis* on mainland China.

The six snail populations from Yunnan and Sichuan provinces, namely *O. h. robertsoni*, were less, and they always fell within a clade in eight phylograms. This supported that snails from Yunnan and Sichuan provinces belong to the same subspecies, *O. hupensis robertsoni*.

Excluding in two single-link trees, Fj-1 and Gx-1, populations always fell within a clade in other six trees. This was dissimilar to the result of allozyme studies (Davis et al., 1995), namely our AFLP analysis data did not support that the Gx-1 population belongs to *O. hupensis hupensis*. In fact, the Gx-1 population is far from the other populations of *O. h. hupensis*, and no snail populations have also been found in the areas between them. The genetic distance D between Gx-1 and Fj-1 snail populations was 0.240, and Nei's unbiased genetic distance was 0.342. There are some morphological similarities between Fj-1 and Gx-1 populations: the shells of these snails are smooth with a varix, and proportionally wider for their length. But the geographical distance between the two snail populations is far, and the shells of Gx-1 snails are thin with weak varix compared with that of Fj-1 population. The genetic distance between Fj-1 and Gx-1 populations is an average level of that among 25 populations. So, should the snails from Guangxi Province be an independent subspecies (i.e., *O. hupensis guangxiensis*) according to the classification of Liu et al. (1981), or be incorporated with snails from Fujian Province into a subspecies? This need further study.

Among the other 17 snail populations, the Js-1 population was an independent clade in the single-link tree using genetic distance D matrix, and the three ribbed populations (Hn-1, Hn-2 and Hn-3) fell within the *O. hupensis robertsoni* subspecies clade in the UPGMA tree using genetic distance D. matrix. However, these snail populations always fell within a clade in the other six trees. These results were similar to the results of allozyme and COI gene sequence, and showed the 17 populations are a subspecies, *O. hupensis hupensis*.

Our results still show the genetic variation between snail populations of *O. h. hupensis* is greater than that between populations of *O. h. robertsoni*. This might indicate there is bigger divergence among *O. h. hupensis*, compared with *O. h. robertsoni*. However, Wilke et al. (2006) found that in their study of 66 individuals from 13 populations of *O. h. robertsoni*, that genetic divergence, using combined COI/LSU rRNA, was an order of magnitude greater than among populations of *O. h. hupensis* (0.042 and 0.085 for different lineages of *O. h. robertsoni;* 0.0097 for other *O. h. subspecies.* But when AFLP was used on 24 individuals, each had a unique fingerprint.

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